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DEPARTMENT OF THE ARMY
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LABORATORY DIAGNOSIS OF HUMAN PSEUDOTUBERCULOSIS

Following is a translation of a German-language article by W. Knapp, Institute for Hygiene of Tübingen University published in Das Arztliche Laboratorium (The Medical Laboratory) Vol. 6, 1960, No. 7, pp. 197-206.

Until recent years, infections with *pasteurella pseudotuberculosis*, the causative agent of pseudotuberculosis occurring in a great number of different animals, appeared to be of no importance in medical-laboratory diagnostics. Among the cases reported as human pseudotuberculosis in the World Literature from 1910 to 1953, only 16 were etiologically confirmed infections with *P. pseudotuberculosis*. However, after *P. pseudotuberculosis* was demonstrated to be the pathogenic agent (Knapp and Masshoff, 1954; Knapp, 1954) of a special form of mesenteric lymphadenitis of adolescents described by Masshoff (1953) as an abscess-forming reticulocytary lymphadenitis, and this infectious disease was noted with increasing frequency both at home and abroad, its laboratory diagnosis began to merit greater attention.

The clinical picture of human pseudotuberculosis is non-characteristic so that a reliable differential diagnosis from other diseases with similar symptoms is possible only by bacteriological and serological tests. Clinical symptoms may correspond to a septic-typhoid affection, an acute or subacute appendicitis and, in some cases, a subacute or chronic enteritis. The symptomatology of an individual case is frequently difficult or impossible to classify under even these syndromes. (1)

Particular attention should be given today in the clinic and the laboratory to a form of human pseudotuberculosis occurring mainly among males between the ages of 5 to 15 years with the clinical symptoms of acute or subacute appendicitis which can generally not be confirmed through biopsy and histology.

Upon surgical intervention, the abdominal cavity shows a clear and serous exudate in varying amounts. The mesenteric lymph nodes, especially

in the ileocecal angle, are infected and swollen and the mesenteric membranes frequently show a reddening which is either diffused or restricted to the environment of the lymph nodes. The distal ileum and cecum also may show hyperemic swelling and edema of the serosa. The appendix generally shows either slight or no infectious changes. Since such affections in the mesenteric lymph nodes may have widely different causes on which we can enter here no more than on the histological picture, the etiological differentiation of the three forms (appendicitis, septic-typoid manifestation, enteritis) remains a task for the bacteriologist.

In the septic-typoid form of human pseudotuberculosis, demonstration of the pathogen has been possible at the early stage and after relapse so far only by means of repeatedly incubated blood cultures. Incubation of *P. pseudotuberculosis* from stools and bile has not yet been possible in man, in contrast to the experimentally infected animal. Disregarding for the moment the absence of respective differential-diagnostic considerations, one reason for this probably is the lack of suitable propagation and selective culture media such as we have available, for instance, for the diagnosis of salmonella. It is also possible that the difficulty of isolating *P. pseudotuberculosis* from stools may be due to a bacterial antagonism or bacteriophagic action and other still unknown factors. However, it has been repeatedly possible to incubate *P. pseudotuberculosis* post mortem from peritoneal exudate, liver and spleen tissue, bile or mesenteric lymph nodes.

In the appendicial form of human pseudotuberculosis, the demonstration of pathogen has so far been possible mainly from infectious changes of the mesenteric lymph nodes, intestinal resection and pus from puncturing as well as from blood specimens in some cases. The frequency of demonstration of the pathogen is mainly conditioned by the knowledge of the surgeons of the bioptic syndrome of this form, the recognition of characteristic changes of lymph nodes and intestinal sections, their interest in the etiological clarification of affections of the mesenteric lymph nodes, the satisfactory collaboration with the medical expert in the laboratory, and the necessity for submitting non-stabilized material for examination. Since it is not infrequent in suspected cases that surgical considerations may make the removal of characteristically inflamed lymph nodes impossible, post-operative procurement of blood specimens is recommended in any suspected case for the demonstration of pathogen and antibodies. Since the demonstration of pathogen causes greater difficulties in our experience than the demonstration of antibodies, we shall first discuss the former in greater detail.

Demonstration of Pathogen

Morphology: *P. pseudotuberculosis* is a gram-negative, pleomorphic, peritrichous rod-shaped bacterium which does not form spores. Form and position of the bacilla are determined by the composition of the nutrient medium as well as by the temperature and duration of incubation. In addition to coccoid and ovoid organisms with a length and width of 0.8-2.0 : 0.8/ μ , there occur slender rods with rounded ends which vary in length and width between

1.5-6.0 : 0.4-0.8 μ . Considered to be a characteristic of the genus, the more intense coloration of the poles (polar staining) is not constant so that it does not possess any particular diagnostic or differential-diagnostic significance in our opinion. By means of staining the flagella and electron-optical examination, it is possible as a rule to demonstrate 3-6 peritrichically arranged flagella in *P. pseudotuberculosis*. Their length is frequently 6 to 9 times that of the bacterial body. Optimum flagella formation takes place in a temperature range of 20-30° C. The diagnostically important demonstration of the motility of *P. pseudotuberculosis* is furnished best by examination of ability for locomotion in Vahlne's U-tube or Bader's U-retort at 22° C.

Cultural Incubation: The cultural incubation of *P. pseudotuberculosis* in liquid or solid nutrient media is not difficult as a rule since the germs exist in pure culture in the specimen. Capable of growing anaerobically, concentration and cultivation are easy in meat broth-peptone solution and a dextrose or "tryptose" bouillon. Frequently, but not always the germs already grow in the first culture on parallel inoculated solid nutrient media. In practice, we therefore recommend the simultaneous inoculation of liquid and solid media. Blood cultures can be prepared with either non-coagulated or coagulated blood through inoculation of liquid media and with non-coagulated blood also by the preparation of blood-agar trays unless the blood specimens are procured directly in dextrose-bouillon "vessels". The liquid blood cultures should be incubated about 8-10 days; transfer to solid nutrient media may be done on the 2nd, 7th and 10th day. All cultures should be prepared in multiples as far as possible so that parallel incubation in aerobic and anaerobic milieu at 22° C and 37° C is possible. Because of the pronounced tendency of *P. pseudotuberculosis* to grow in "rough" form at 37° C and to lose motility, antigen analysis or examination for motility is frequently possible for germs of a first culture only if they are cultivated at 22° C. It should be remembered moreover that in individual cases first cultures will incubate only at 22° C and/or in anaerobic milieu so that cultural demonstration of *P. pseudotuberculosis* is recommended by both aerobic and anaerobic culture at 22° and 37° C.

In such liquid milieu as meat broth, peptone or tryptose bouillon, *P. pseudotuberculosis* at 37 and 22° C produces a slight diffused turbidity of the culture medium within 24 hours. After incubation of the cultures for 2 or more days, a slimy or scummy or crumbly, relatively firmly adhering sediment is precipitated while the bouillon becomes clear again. Mold formation is observed only infrequently. Rough forms develop more frequently at 37° than at 22° C, grow as a function of the intensity of dissociation in rough form with and without turbidity of the bouillon as a lumpy or flocculated sediment. An addition of serum or dextrose to the bouillon does not necessarily promote growth but more intense growth and quicker propagation are observed in tryptose bouillon.

P. pseudotuberculosis grows on nutrient agar within 24 hours in the form of clear transparent colonies with a diameter of about 0.25-1.0 mm. Upon continued incubation, the colonies assume an opaque milky turbidity

and a whitish to yellowish-gray color. Mature colonies (1.0-2.0 mm in diameter) are of slimy or soft consistence at 22° C and slightly raised in the center. Their surface is smooth or slightly granulated, has a moist reflection and the colony border is smooth and regular. Colonies grown at 37° C or on unfavorable culture media frequently appear dry and flat which is the sign of beginning dissociation in rough form; the clearly granulated surface gives a mat reflection or is dull. The colony border may be irregular or flattened like a seam (formation of seam border) which constitutes a particular important diagnostic characteristic for *P. pseudotuberculosis*. The same appearance of a colony is presented also on blood agar cultures but without simultaneous hemolysis.

It is generally possible without difficulty to isolate *P. pseudotuberculosis* from specimens on blood or nutrient agar cultures. For rapid differential diagnosis and differentiation from lactose-fermenting germs, it is recommended to effect simultaneous inoculation of lactose-sulphite agar (Endo's culture). This medium appears to be especially favorable for growth of *P. pseudotuberculosis* at 22° C. The initially light and transparent and subsequently slightly turbid smooth colonies reach the same size within 48 hours as on nutrient and blood agar media. The color of the medium remains unchanged. Cultures incubated at 37° C generally show the rough form. With Endo's agar, the colonies show a broad seam border with radial striping more clearly than on nutrient and blood agar.

Growth of pure cultures on desoxycholate-citrate agar at 22 and 37° C is inhibited only slightly in comparison with the intensity of growth on nutrient, blood and Endo's agar but dissociation in rough form is greater at 37° C. So far we have been unable to isolate *P. pseudotuberculosis* from human specimens on this medium -- in contrast to Endo's agar -- but the number of cases investigated is not yet sufficient to form a definite opinion on the value of this medium in the cultural diagnosis of human pseudotuberculosis. On bismuth-sulphite agar (Wilson and Blair) strongly inoculated with pure cultures, incubation of the cultures at 22° C produced only very slight growth of a few strains.

Whether the culture media (tetrathionate bouillon, selenite broth, desoxycholate-citrate agar, etc.) tested for enrichment and differentiation in the diagnosis of salmonella and/or shigella will be found appropriate for the diagnosis of human and animal pseudotuberculosis will remain an open question until experience on larger experimental material has been gathered. In our observations, inoculation of the selenite broth (Leifson, 1936) with pure cultures of *P. pseudotuberculosis* is followed by a perceptible germ reduction.

Cultural-Biochemical Examination

A reliable cultural diagnosis of suspected colonies and/or a differential-diagnostic delimitation of *P. pseudotuberculosis* within the pasteurella group and from germs of the salmonella group is possible on the basis of our present knowledge only with the aid of various metabolic tests. A

polytropic medium making it possible to reliably demonstrate anabolic processes ("Stoffwechselleistung") characteristic for *P. pseudotuberculosis* has not yet been found. Suspected colonies are examined serologically, and in addition, culturally in a "limited diversified range" ("kleine bunte Reihe") which simultaneously makes possible a differential-diagnostic delimitation from *P. multocida*, *P. pestis* and germs of the salmonella group (cf. table 1)

Table 1
Cultural-Biochemical Tests

Cultural Medium	<i>Pasteurella</i>	<i>pseu. tuber. losis</i>	<i>multocida</i>	<i>pestis</i>	Salmonella Group
Mobility	+	-	-	-	+
Melibiose	+	-	-	-	+
	(-)				(-)
Salicin	+	-	+	-	-
	(-)	(+)	(-)	(+)	
Rhamnose	+	-	-	-	+
			(+)	(-)	
Sorbit	-	+	-	-	+
	+	(-)	(+)		
Saccharose	-	+	-	-	-
	(+)		(+)		
Urea	+	+	-	-	-
Hydrogen Sulphide	(+) very slight	+	-	-	+
					(-)
Indole	-	+	-	-	-

Note: (+) = infrequent exceptions; (-) = infrequent exceptions

1 - acidification without gas formation

Differential-diagnostic differences of *P. pseudotuberculosis* from *P. Multocida* and *P. Pestis* are in particular its motility and the ability to cleave melibiose and rhamnose without formation of gas and, from germs of the salmonella group, the rapid cleavage of urea, the almost uniform acidification of salicin, the frequently absent acidification of sorbit and the generally not or only very slightly demonstrable formation of hydrogen sulphide in iron agar (Kligler) and "SIM" agar.

The metabolism tests in the "small diversified range" must be complemented through further metabolism investigations if the result of the investigations listed in table 1 is not definitive and/or serological classification of suspected colonies is not possible. However, the individual metabolic reactions ("Stoffwechselleistungen") of *P. pseudotuberculosis* can be discussed only very briefly.

P. pseudotuberculosis cleaves arabinose, galactose, glucose, glycerine, levulose, maltose, mannite, melibiose, rhamnose, trehalose and xylose with acidification but without formation of gas whereas erythrone, inositol and lactose are not decomposed. The enzymatic reaction of individual strains to amygdaline, dextrine, raffinose and sorbitol is not uniform. The absence of acidification of melibiose, adonit and salicin as well as the cleavage of dulcitol, inulin and saccharose represent very infrequent exceptions. Of other biochemical reactions differing in intensity -- occasionally totally absent in individual cases --, rapid reduction of nitrate and methylene blue, formation of ammonia, decomposition of urea, and a positive reaction of methyl red and catalase may be mentioned. Formation of hydrogen sulfide is absent or only faintly demonstrable and indole and/or Voges-Proskauer reactions are negative.

Serological Examination (Antigen Analysis)

P. pseudotuberculosis possesses thermostable O and thermolabile H antigens. In addition to thermostable O antigens, thermolabile O antigens probably also occur for individual types but the investigations on this are not yet terminated. Antigen analysis is provisionally restricted to the demonstration of the thermostable-type specific antigens I-V and that of the thermolabile H antigen a common to *P. pseudotuberculosis*-type I, II, III and V and/or the H antigen b recognized in two strains of type IV (No. 32 and 190). A determination of the subtypes A and B recognized for type I, II and IV has no significance for routine diagnosis. Serological examination can be carried out by means of the slide or tube agglutination if the strains to be investigated exist in the smooth form and do not agglutinate spontaneously.

In the serological examination and determination of type of suspected cultures, particular attention must be given to the antigens partially common to *P. pseudotuberculosis* type II and the O factors 4 and 27 of the salmonella-B subgroup and/or to *P. pseudotuberculosis* type IV and the O factors 9 and 46 of the salmonella-D subgroup. Minor antigen relations exist moreover between *P. pseudotuberculosis* type IV and the O factor 14 of the salmonella-H subgroup (Knapp, 1960).

For the types of *P. pseudotuberculosis*, it is possible to provisionally develop the following antigen diagram by means of the agglutination method (cf. table 2). The antigen relations to *P. Pastis* can be disregarded in this context because infections from plague play no role in Germany diagnostically or differential-diagnostically.

Table 2
Antigen Diagram of *P. Pseudotuberculosis*

Type	Subtype	Rough Antigens	Body Antigens			Flagella Antigens
			Type-Specific Antigens	Subtype-Specific Antigens		
I	I A	1	2	3		a
	I B	1	2	4		
II*	II A	1	5	6		a
	II B	1	5	7		
III	- -	1	6	-		a
IV*	IV A	1	9	11		b
	IV B	1	9	12		
V	- -	1	10	-		a

* = Antigen relations to salmonella-B and D subgroups not considered in table.

In order to exclude cross-reactions with salmonella germs of the subgroups mentioned, saturated-type sera are utilized for the agglutination tests. Cross-reactions between *P. pseudotuberculosis* type II and strains of the salmonella-B subgroup are absent when utilizing subtype-specific sera of pasteurella type II-A and II-B or type II-antisera saturated. e.g., with Salm. reading (4, 12), Salm. abortus equi (4, 12) and Salm. schleissheim (4, 12, 27), and are also absent between *P. pseudotuberculosis* type IV and the salmonella-D and -H subgroups when utilizing type IV-antisera saturated with Salm. enteritidis (1, 9, 12) or Salm. typhi (9, 12) as well as Salm. Strassburg (9, 46) and Salm. Carrau (6, 14, 24). (The type sera are obtained from rabbits immunized with suspensions of culture heat-treated for 180 min. at 100° C.).

The demonstration of the H antigens is carried out in H sera obtained by saturation of complete sera with boiled homologous cultures or in non-saturated OH sera obtained from rabbits immunized with highly mobile strains of heterologous types.

The type of the H agglutinate is finely to coarsely flocculate or compactly viscous and that of the O agglutinate is finely or coarsely granulate in tube agglutination. Since living or carefully killed strains in O sera also show a compactly viscous agglutinate, the interpretation as O or H agglutination is frequently difficult or impossible -- even with the Widal reaction -- if the point in time of its onset is not taken into consideration.

The greater number of the strains so far demonstrated in man and animals belongs to type I..

The following procedure has proved itself for us in the antigen analysis of suspected strains by means of the test agglutination on the slide (PA):

Agglutination in OH collective serum type I-V
PA positive:

Agglutination in O collective serum type I-V
(agglutination frequently weaker than in OH collective serum).
PA negative: possible existence of new O type with H antigen a or b.

PA positive:

Agglutination in: O type sera type I-V
Positive PA in O type sera type II and IV

Agglutination in: Saturated O sera
Type II and IV to exclude cross reactions with salmonella-B and D subgroups.

H-Antigen determination:

Agglutination in: H Ser a and/or o (This agglutination should be carried out with highly mobile cultures incubated at 22°C).

Diagnostic Animal Test

The most suitable animal is the guinea pig although spontaneous infections do occur which should be taken into account in the evaluation of positive animal tests. At least two animals are injected with the same specimen to be tested. After intramuscular and/or subcutaneous injection with infectious specimens (unless the infection remains locally restricted), death occurs as a rule within 2 to 6 weeks and after about 7-30 days subsequent to intraperitoneal injection. Upon autopsy, liver and spleen show nodular foci of different size and number similar to the tuberculosis of the guinea pig whereas the intestine frequently does not show any macroscopically visible foci.

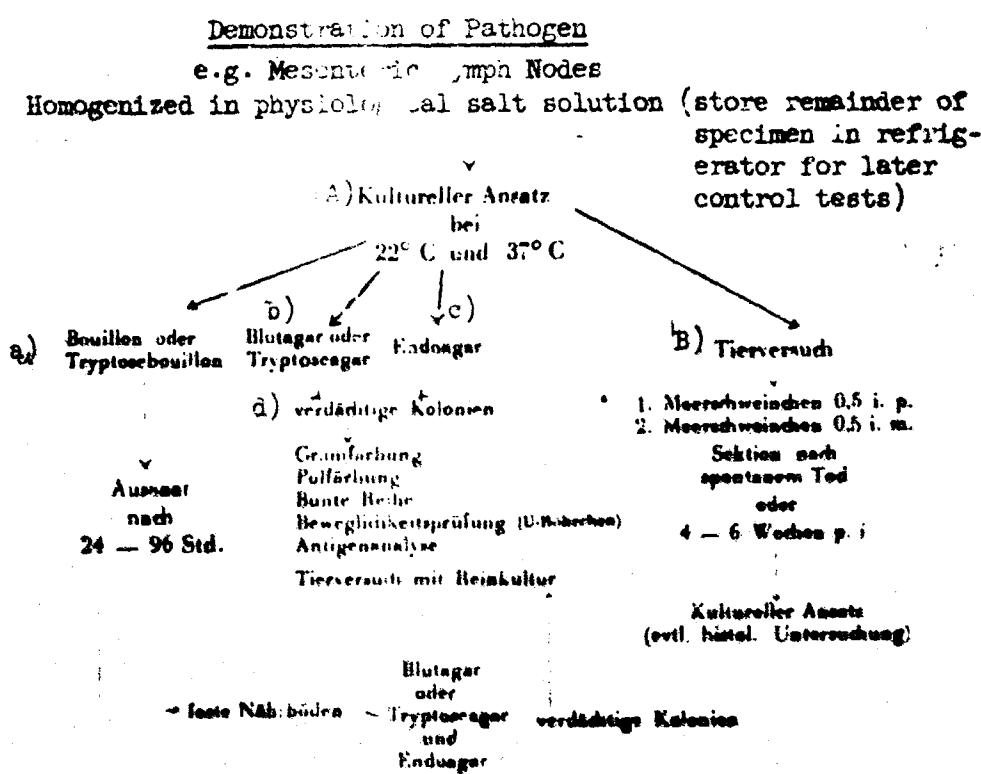
In some animals, we find either only enlarged mesenteric lymph nodes and a serous or hemorrhagic exudate, or the picture of a serofibrinous pericarditis and peritonitis is in the foreground of the macroscopic changes.

Diagnostic animal tests with blood specimens or crushed material from surgery or autopsy should be instituted until antibody demonstration is positive and culture negative. A negative result of the animal experiment with positive culture does not contradict the result of the cultural, biochemical and serological examinations because differences in pathogenicity of the several types of *P. pseudotuberculosis* -- disregarding differences in the virulence of individual strains of the same type -- are

observed in the guinea pig. It would seem that the pathogenicity in the guinea pig of strains of type IV and V is very minor in contrast to the strains of type I, II and III. The only two strains of *P. pseudotuberculosis* type V incubated by us from mesenteric lymph nodes were as nonpathogenic in the guinea pig as the two strains of type IV incubated by Thal. However, the small number of strains investigated does not permit any definite conclusion from these observations.

Animal tests are not made if the cultural and serological examinations have been negative or the culture has been positive.

The routine procedure for demonstration of pathogen developed by us can be schematized as follows:



Key: A) Culture Tests: Incubate at 22 and 37°C in:

a) meat broth or tryptose bouillon - after 24-96 hours, seed in solid culture media (blood agar or tryptose and Endo's agar) - suspected colonies - animal test with pure culture;

b) blood agar or tryptose agar - suspected colonies;

c) Endo's agar - suspected colonies

d) Suspected colonies: Subject to gram staining; polar staining; diversified range test; mobility test (U-tube); antigen analysis.

B) Animal Tests: Inject one guinea pig with 0.5 intraperitoneal and one guinea pig with 0.5 intramuscular - autopsy upon exitus or 4-6 weeks after infection - culture test (eventually histological examination).

III. Demonstration of Antibody

The demonstration of antibody (Widal reaction) is made with agar culture suspensions of pasteurella strains type I-V which are preferably living or have been carefully killed by 0.25% phenol and/or formalin. Living antigens must be prepared freshly each time but antigens killed with phenol or formalin can be utilized for several months.

In regard to method, it should be taken into account in the preparation of the Widal reaction that, as already mentioned, the agglutinates of the living antigens frequently do not show the picture familiar from the salmonella-Widal reaction and even test strains which appear stable in physiological or 3.5% sodium-chloride solution and/or in boiling, may agglutinize spontaneously in only slightly diluted sera whereas the spontaneous agglutination is absent with increasing serum dilution. Each Widal reaction should therefore be paralleled by a sodium-chloride and serum control where the concentration of the control serum should correspond to the first dilution of the patient serum. Frequently there must be taken into consideration a difference in the agglutinability of various strains of the same type so that the Widal reaction should be prepared preferably with several strains of the same type incubated at 22° C. In some cases, only the characteristic strain in the patient serum is agglutinized whereas the antibody demonstration with other strains is absent.

The agglutinins against P. pseudotuberculosis type I observed most frequently are demonstrated as a rule already with the occurrence of the first disease symptoms of the appendicinal form. Titers lie between 1:80-1:10240 -- generally between 1:160-1:640 -- of the serum dilution. Infections with P. pseudotuberculosis type II, III, IV and V appear to occur only infrequently in man. In most of the patient sera of the appendicinal form of human pseudotuberculosis, boiled antigens are either not at all or only slightly agglutinized (in contrast to living or carefully killed antigens) although the boiled homologous antigens may lead to a saturation of the patient sera. In regard to an agglutination of strains type II and IV, their already mentioned antigen relation to the salmonella-B and D subgroups must be taken into account. Their agglutination does not prove the existence of an infection with P. pseudotuberculosis. In doubtful cases, serum saturation must be carried out with salmonella strains of the subgroups to which antigen relations exist. Antibody demonstration may also be attempted through the complement fixation test. According to joint investigation with Steuer (1956) the agglutination test is superior to the complement fixation test. If the demonstration of complement-fixing antibodies is successful in the patient sera, their titer does not reach the magnitude of the agglutination titer. The precipitation test is without significance for antibody demonstration in patient sera.

The points essential for antibody demonstration can be briefly reduced as follows:

Patient serum: dilution beginning with 1:20

Antigens: 1) Live or carefully killed permanent antigens (type I-V) (stable when boiled);
2) Boiled antigens (180 min, 100° C, type I-V) (elutions after 48 hours of cultures incubated at 22° C)

Control: Antigens type I-V plus physiological salt solutions;
Antigens type I-V plus standard serum (1:20)

Preparation: 16-20 hours water bath at 52° C

Reading of Widal reaction after: 4 and/or 16-20 hours

Cross reactions: P. pseudotuberculosis type II and O factor 4 and 27 of the salmonella-B subgroup; P. pseudotuberculosis type IV and O factor 9, 46 and/or 14 of salmonella-D and/or H subgroup

Eventual serum saturation If agglutination of P. pseudotuberculosis type II and IV in patient sera.

The foregoing may create the impression that laboratory diagnosis of human pseudotuberculosis no longer leaves any questions open. However, this goal has not yet been reached. Still unsatisfactory is, e.g. due to the lack of suitable culture media, the demonstration of pathogen from stool specimens and with this the bacteriological diagnosis of the septic, typhoid and the enteritic form of pseudotuberculosis which probably occurs more frequently than recognized so far. Antigen analysis and antibody demonstration also still pose a number of puzzles which cannot be discussed in this frame. It is our task to answer these still open questions with the help of a larger wealth of observation which can be obtained only through a comprehensive collaboration between clinicians, bacteriologists and pathologists.

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